Non-sterile heterotrophic cultivation of native wastewater yeast and microalgae for integrated municipal wastewater treatment and bioethanol production

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Abstract

Currently over 80% of wastewater generated globally, is discharged into surface waters without adequate treatment. Major environmental and public health risks associated with such releases are particularly prevalent in developing countries where the infrastructure and financing for effective treatment is lacking. Novel low cost integrated wastewater treatment and biorefinery processes could provide a sustainable solution. This study investigated, for the first time, the feasibility of simultaneous wastewater treatment and bioethanol production in non-sterile, heterotrophic bioreactors using either microalgae, wild yeast, or a co-culture of microalgae and wild yeast. *Scenedesmus* sp. are known to achieve high biomass concentrations under sterile heterotrophic conditions. However, under the non-sterile conditions proposed, relatively low nutrient removal rates (60% nitrate, 53% total ammonia nitrogen (TAN) and 46% orthophosphate) and biomass yields (0.98 ± 0.10 g/L) were achieved. Wastewater grown microalgae and yeast co-cultures achieved high nutrient removal rates (96% nitrate, 100% TAN and 93% orthophosphate). Wastewater grown yeast cultures produced consistently promising results, achieving high biomass concentrations of 3.7±0.1 and 4.2±0.1 g/L along with 100% nitrate, 100% TAN and 92.6% orthophosphate removal. Yeast provided the additional advantage of

aerobic fermentation, possibly allowing integrated wastewater treatment and bioethanol production.

Keywords: Wild yeast, Sewage treatment, Non-sterile microalgae growth, Nitrate removal, Phosphorous removal, Crabtree positive yeast

1. Introduction

Inadequate sewage treatment and its negative effects on health, economy and the environment is a major challenge for developing countries. Although representative data on the global generation, collection and treatment of wastewater is not well published; The United Nations, 2017 [1] estimate that over 80% of all collected wastewater is discharged without adequate treatment. The release of untreated water is particularly prevalent in developing countries where infrastructure, technical resources and financing for effective treatment is lacking. Such is the case in Mexico, where only 47.5% and 28.8% of municipal and industrial wastewater were treated in 2012 [2]. In an attempt to resolve this, a nation-wide water program was established to improve access to high quality drinking water. The program aims to prioritise individuals within the most vulnerable populations through the modernisation of wastewater treatment plants [2]. However, the high cost of operating wastewater treatment plants has hindered progress to date. The adoption of novel technologies that generate revenue from the waste could provide a sustainable solution.

Since the 1950's, there has been great interest in microalgae wastewater treatment. Microalgae are a diverse group of unicellular microorganisms that efficiently convert inorganic carbon into biomass via photosynthesis whilst consuming nutrients such as nitrates and phosphates. They thrive in a range of environments including wastewater and the resulting microalgal lipids, carbohydrates and proteins provide feedstocks for biodiesel, bioethanol, bioplastic or industrial

pigment products [3]. Recent studies have concluded that heterotrophic growth has the potential to overcome some of the major challenges associated with autotrophic cultivation in high rate algal ponds. Unlike autotrophic growth, heterotrophic systems are not limited by light penetration or local weather conditions meaning higher productivities and biomass concentrations can be achieved all year round at large scale. In addition, growth can be carried out in practically any fermenter eliminating the need for large land areas [3]. Although the ability to switch nutritional mode based on substrate availability and light conditions is limited to relatively few microalgae species, *Scenedesmus* sp. has been successfully grown under heterotrophic conditions, achieving a maximum biomass concentration of 3.46 g/L [4], in six days when supplemented with 10 g/L of glucose. However, these results along with many others reported in literature were obtained from microalgae cultivated under sterile conditions. Under non-sterile heterotrophic conditions, it has been reported that microalgae are unable to compete with other microorganisms such as bacteria and yeast in the culture medium due to their inferior glucose assimilation efficiency [5]. Two microalgae strains, *Chlorella protothecoides* and *Chlamydomonas acidophila* were unable to compete with neutral and acidic bacteria, respectively, in wastewater supplemented with a range of glucose concentrations up to 10 g/L [5]. This suggests a sterilisation process such as autoclaving or microfiltration would be required to achieve sufficient sterility for successful heterotrophic microalgae growth in municipal wastewater. As autoclaving is an energy intensive process the high associated costs would render the process economically infeasible.

Whilst yeast have wide spread applications in the brewing, baking and pharmaceutical industries; their application in the wastewater treatment industry has not been fully explored possibly due to the non- sterile conditions. Despite this, phosphorus typically makes up 3-5% of a yeast cell's dry weight [6] significantly greater than the typical content of 0.87% for microalgae [7].

The nitrogen content of yeast is also higher at 10% [6] compared to around 6% for microalgae [7]. This suggests that yeast may be capable of removing, compared to microalgae, a greater proportion of the nutrients from wastewater. Yeast also present the major advantage of good settling and dewatering properties allowing cost effective removal of biomass. Traditional wastewater treatment plants (WWTP) use activated sludge to remove organic matter. Yeast provide the unique advantage of simultaneous chemical oxygen demand (COD) and nutrient removal. A study by Lim, Kim and Hwang [8] found that yeast were able to reduce the COD of industrial wastewater by 70% in 6.3 hours,. The dual application of yeast to remove organic matter from municipal wastewater and eliminate the need for additional nutrient removal steps, could minimise operational costs associated with wastewater treatment. Whilst the capability of yeast to dramatically reduce the organic load of industrial wastewaters has been reported, the feasibility of combined COD and nutrient removal by yeast has not been explored. Following wastewater treatment, residual yeast biomass could be utilised for bioethanol production. Bioethanol is the most widely used biofuel owing to its ability to greatly reduce crude oil consumption and atmospheric pollution. Inoculum preparation for corn ethanol production typically requires additional fermentable sugars typically derived from the food crop. Hence, the use of yeast cells produced during wastewater treatment for ethanol biosynthesis would reduce the food crop requirements. Furthermore, the use of treated wastewater effluent would eliminate the need for fresh water for bioethanol production as shown in Ramchandran, Singh, Rajagopalan and Strathmann [9]. Synergically, the economic feasibility of large-scale wastewater treatment would be improved through the generation of a valuable bioethanol product. However, as the capability of yeast to both successfully treat wastewater and produce high quality bioethanol has not been investigated, further research is required.

Interest is emerging in the use of combined symbiotic microalgae and yeast cultivations for improved integrated wastewater treatment and biofuel production [10-12]. Microalgae generate oxygen which is utilised by yeast to assimilate carbon substrates, the yeast subsequently release CO_2 promoting microalgal photosynthesis. As the negative charge of microalgal cells prevents self-flocculation, a costly and/or energy intensive flocculation step is required to recover valuable biomass. Fungal species which possess effective flocculation properties have been found to effectively trap microalgae cells aiding biomass recovery [13]. Iasimone, Zuccaro, D'Oriano, Franci, Galdiero, Pirozzi, De Felice and Pirozzi [11], found a mixture of an oleaginous yeast strain, *Lipomyces starkeyi*, and the native wastewater microalga, *Scenedesmus* sp. and *Chlorella* sp. reduced the lag phase associated with microalgae growth by three days compared to the microalgae alone. This was attributed to CO_2 enrichment resulting from yeast respiration. Alkalinisation during microalgae growth increased the pH of the culture medium to 10.9 which had beneficial antibacterial and antifungal effects leading to a predominantly microalgal culture after 14 days of cultivation. Although biomass and lipid productivity increased, the final biomass and lipid concentrations were around 300 and 45mg/L, respectively. As a result of the aforementioned high costs associated with the running of raceway ponds, such yields are likely to be insufficient for sustainable combined wastewater treatment and biodiesel production. The cultivation of a mixture of the microalgae and yeast strains, *Chlorella vulgaris* and *Yarrowia lipolytica* by Qin, Wei, Wang and Alam [12], achieved significantly higher biomass and lipid yields of up to 1.53 g/L and 187 mg/L, respectively. However, synthetic wastewater and an energy intensive sterilisation process were employed.

The aim of this study was to investigate, for the first time, the feasibility of novel non-sterile integrated municipal wastewater treatment combined with bioethanol production for improved sustainability. Both, microalgae (mostly *Scenedesmus* sp.) and a consortium of yeast isolated

from municipal wastewater were grown under non-sterile heterotrophic conditions. The effectivity of co-cultivation or single strain cultivation was assessed in terms of strain growth, COD and nutrient removal during wastewater treatment.

2. Materials and methods

Several wastewater treatment processes were studied using wild yeast and microalgae. Cultures were tested under non-sterile heterotrophic conditions to evaluate wastewater treatment and the simultaneous production of high value biomass. Three systems were investigated sequentially in batch bioreactors (Table 2): 1) Cultures with a predominance of microalgae; 2) Co-cultures of microalgae and wild yeast; 3) Cultures with a predominance of wild yeast. Nutrient removal efficiency and biomass accumulation were monitored for each of the aforementioned culture systems over a period of eight days.

2.1. Wastewater preparation and characterisation

Municipal wastewater provided the culture medium in all bioreactors, this was collected from the effluent of a treatment plant located on the central campus of the Universidad Nacional Autónoma de Mexico. Prior to collection, the wastewater underwent primary and secondary treatment. This involved two sequential bio-towers, in the first organic matter was removed whilst in the second partial nitrification was performed to reduce the organic load.

Culture medium preparation involved the filtration of wastewater samples on site to remove large suspended solids using a filter with an 8µm pore size. The filtered samples were then stored at 4°C in 20L high density polyethylene (HDPE) containers until use. As the initial concentration of nutrients varied depending on collection time, samples of the filtered wastewater were characterised according to the following standard methods.

Nitrate and orthophosphate concentrations were measured using the HACH® cadmium reduction and amino acid methods, respectively [14], whilst COD was monitored using the HACH® reactor digestion method [14]. To avoid interference, due to the colorimetric nature of the method, each sample was filtered using a Whatman® GF/A glass fibre filter with a 1.6 μm pore size prior to the addition of reagents and measurement using a HACH® DR3900 Spectrophotometer. Total ammonia nitrogen (TAN) concentration was determined using a modified Total Kjeldahl Nitrogen (TKN) method [15] using a BÜCHI B-324 Distillation Unit. In aqueous solution, ammonia exists as an equilibrium of its two principal forms, unionised ammonia (NH_3) and ammonium ions (NH_4^+). The relative concentrations of these depend on temperature and pH. TAN was given by the sum of the NH_3 and NH_4^+ concentrations. The standard TKN method quantifies both inorganic and organic nitrogen. Both microalgae and yeast are rich in organic nitrogen which could lead to false positive results. It was therefore necessary to omit the organic nitrogen digestion step to facilitate measurement of the TAN concentration.

Finally, the carbohydrate concentration was monitored using the phenol-sulphuric acid method [16]. A HACH® DR3900 Spectrophotometer was used to measure absorbance at 490nm; and concentrations were determined from a calibration curve produced using known concentrations of glucose. Filtration of the sample using a Whatman® GF/A glass fibre filter with a 1.6μm pore size was also performed prior to reagent addition to remove cells containing internal carbohydrates and ensure the concentration corresponded to that of the culture medium. Summary of wastewater characteristics obtained from four wastewater samples collected over an eight week period are shown in Table 1.

Table 1: ***Characterisation of municipal wastewater culture medium.*** *Values shown are mean values ± standard deviation obtained for four samples collected over an eight week period.*

Parameter	Value
Nitrate Concentration (mg/L)	15.0 ± 4.9
Orthophosphate Concentration (mg/L)	67.5 ± 19.5
TAN Concentration (mg/L)	133.1 ± 13.9
Chemical Oxygen Demand (COD) (mg/L)	95 ± 24.7
Total Suspended Solids (TSS) (mg/L)	47.9 ± 35.8
pH	7.5
Carbohydrates (mg/L)	5.7 ± 3.9

2.2. Experimental setup and analytical procedure

Wastewater treatment performance in each of the culture systems (Table 2) was studied in 5L batch polyethylene terephthalate (PET) bioreactors (L:14 cm; W:12 cm; H: 25 cm) wrapped in aluminium foil to maintain heterotrophic conditions with a working volume of 60%. Air was supplied continuously using an ELITE 802 dual outlet air pump at a rate of 0.5 VVM. Initial trials found that an additional carbon source was required to sustain growth. Glucose was selected to increase the low COD of municipal wastewater (Table 1) as it is the preferred sugar for the microorganisms used in this study. Several concentrations between 0 and 10 g/L were initially investigated and 10 g/L was ultimately chosen for subsequent experiments (Table 2). Duplicate measurements of nitrate, orthophosphate and, TAN were taken for all experiments. Biomass concentration was measured as total suspended solids (TSS) following the APHA 2540 D standard method [17], cultures were agitated thoroughly prior to taking the measurement to ensure heterogeneity and break up any microbial flocs. Microscope images were taken using a Zeiss Axio Lab A1 microscope with a 0.5x camera adapter. Objective values of 63 and 100X, equivalent to total zoom values of 31.5 and 50X, respectively, were used.

Table 2 ***Cultivation systems evaluated for wastewater treatment***

Number (#)	**Cultivation Name**	**Starting inoculum (concentration)**	**Glucose (g/L)**	**Final pH**	**Time (days)**	**# B.E.**
1	Microalgae, Batch 1	Microalgae plus wastewater microbes (total of 0.2 ± 0.02 g/L)	0-20	7-8	8	2
2	Microalgae/Yeast, Batch 1	Microalgae from #1 (0.4 ± 0.03 g/L) plus wastewater with yeast (0.03 g/L) giving a total of 0.2 g/L (94 wt% microalgae, 6 wt% yeast)	10,20	7	7	3
3	Yeast, Batch 1	Co-culture from #2 (1.9 g/L) plus wastewater giving a total of 0.2 g/L	10	4-5	8	3
4	Yeast, Batch 2	Culture from #3 (3.7 g/L) plus wastewater giving a total of 0.2 g/L	10	4-5	8	3
5	Yeast, Batch 3	Culture from #4 (4.2 g/L) plus wastewater giving a total of 0.2 g/L	10	4-5	2	3

#BE: Number of biological experimental replicates run in parallel. All cultivations had controls run in parallel.

2.2.1. Microalgae culture preparation

The microalgae used to inoculate the heterotrophic bioreactors were pre-adapted to wastewater as per [18] using *Scenedesmus obliquus* (Turpin) Kützing as provided by Instituto Politécnico Nacional, Mexico City, México. Bioreactors were run as per Table 2, #1 including control cultures containing just wastewater, without microalgae or glucose. The inoculum fluid was mixed with filtered wastewater (1:2 v/v) giving each reactor an initial biomass concentration of 0.2 ± 0.02 g/L. Following inoculation, the cultures contained 15.1 ± 1.3 mg/L nitrates, 81.0 ± 0.7 mg/L TAN and 44.2 ± 1.2 mg/L orthophosphates. The pH of each culture was also measured on each of the eight days and volumes of 6N NaOH added, as required to maintain the pH of the culture medium within the optimal range for microalgae between 7 and 8. The results of this first investigation indicated that a concentration of 10 g/L glucose was optimal for biomass production.

2.2.2. Microalgae and yeast co-culture preparation

Concentrated microalgae cultures were used to prepare the inoculum for the microalgae and yeast co-cultures (Table 2, #2) and additional control reactors containing only wastewater supplemented with 10g/L of glucose. The cultures resulting from microalgae experiments were mixed producing a stock culture with 0.4 ± 0.03 g/L of biomass, which was then added to wastewater containing 0.03 g/L of yeast (1:1 v/v). The resulting cultures had an initial total biomass concentration of 0.2 g/L (94 wt% microalgae, 6 wt% yeast) along with 66.2 ± 4.9 mg/L nitrates, 76.2 ± 0.3 mg/L TAN and 78.5 ± 1.0 mg/L orthophosphates. The pH of each culture was measured each day and volumes of 6N NaOH added, as required, to maintain neutral pH. Relative microalgae growth was assessed at the end of the fermentations by microscopy. Cultures were mixed thoroughly to ensure heterogeneity before a series of images were taken for samples of triplicate bioreactors. The mean number of microalgae cells was calculated for each of the glucose concentrations studied.

225

226 ### 2.2.3. Yeast culture preparation

227 In the third system, biomass resulting from replicate co-cultures was mixed, giving a stock
228 culture with a TSS concentration of 3.5 g/L. This was then added to wastewater in a 1:14 v/v
229 inoculum:wastewater ratio. Following inoculation each reactor contained 16.2 ± 1.1 mg/L of
230 nitrates, 44.5 ± 0.6 mg/L of orthophosphates, 141.2 ± 1.2 mg/L of TAN, 10016 ± 1.9 mg/L of
231 carbohydrates and 10821 ± 5.3 mg/L of COD. Each tested batch was run as per Table 2, #3-5,
232 plus additional control bioreactors containing wastewater supplemented with 10 g/L of glucose.
233 After the first batch, ethanol concentration was monitored in subsequent batches and this was
234 achieved by distillation using a BÜCHI B-324 Distillation Unit following the manufacturer's
235 instructions [19].

236

237 ## 2.3. Statistical analysis

238 Statistical analysis was performed using Minitab 17 statistical software and MATLAB R2017a.
239 T-tests were performed in Minitab® using a significance level of 0.05 to analyse and compare
240 results from different parallel batches. The null hypothesis considered that there was no signif-
241 icant difference between the cultures, hence if $p \leq 0.05$ the null hypothesis was rejected. The
242 wastewater composition varied depending on time of collection. A test for equal variance was
243 therefore performed using Minitab to assess the repeatability of yeast growth between batches
244 of replicates. Linear regression was also performed using MATLAB by plotting results of the
245 first batch against those of the second batch. The resulting R^2 values for biomass, orthophos-
246 phate, carbohydrate and TAN were 0.9535, 0.9842, 0.9979 and 0.987 respectively indicating a
247 high level of repeatability between batches.

248

249 ## 2.4. Yeast identification: DNA extraction, sequencing methods and analysis

Dried samples of microalgae and yeast co-cultures underwent DNA extraction. 200 mg of dried sample was weighed into a Lysing Matirx E tube and hydrated with 300 μL of molecular-grade H_2O. DNA was then extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) as per the manufacturer's instructions. Extracted DNA was subsequently diluted 1:10 with molecular-grade H_2O in order to dilute out any PCR-inhibiting substances that were co-extracted. Next, diluted template DNA was quantified using a Qubit 3.0 fluorometer using the dsDNA HS Assay Kit (Life Technologies, USA) according to the manufacturer's instructions. Diluted template DNA then underwent PCR targeting the fungal ITS1 region, using the primers ITS1f (5` – CTTGGTCATTTAGAGGAAGTAA) [20] and ITS2 (5` – GCTGCGTTCTTCATCGATGC) [21] as per the EMP Protocol [20]. The PCR involved a 10 μL reaction comprised of: 9 μL of MegaMix Blue (Microzone, UK), which contained *taq* polymerase, dNTPs, $MgCl^{2-}$, buffer, and agarose loading dye; 0.25 μL of each primer (100 pM concentration) and; 0.5 μL of 1:10 diluted template DNA. PCR conditions were as follows: Initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 68°C for 30 seconds, and a final elongation step of 68°C for 10 minutes. PCR amplification was performed on a Techne 512 thermocycler. Procedural positive was DNA from *Trichoderma reesei* (DSMZ no. 768) and procedural negative was molecular grade water. Following PCR, amplicon product and fragment size checks were performed by agarose gel electrophoresis. The agarose gel was comprised of 1 g of agarose (UltraPure, Invitogen, USA), 99 mL of 1 x TAE (tris-acetate-EDTA) buffer and 1.6 μL of ethidium bromide (Bio-Rad, USA). As the MegaMix Blue reagent contained loading dye it was not necessary to add any additional dye. Marker lanes were loaded with 2.5 μL of HyperLadder 50bp (BioLine, UK), which contained DNA fragments of varying sizes, ranging from 50 bp to 2.1 kbp, to determine PCR amplicon fragment size. Electrophoresis was performed for 45 minutes at 100 V. Gels were visualised using a UV MultiDoc-It™ Imaging System (UVP, USA).

Diluted template DNA was then submitted for sequencing on the Illumina MiSeq platform (Illumina, USA) at the NUOMICs Group (Northumbria University, UK). Sequencing was performed targeting the fungal ITS1 region following the EMP Protocol [20]. Sequencing produced 255,617 paired-end reads in a demultiplexed FASTQ file. Due to inadequate quality of the reverse reads, only the forward single-end reads were used in subsequent analysis. Sequencing analysis was performed using DADA2 version 1.12 [22] in R version 3.6.0 [23]. Sequences were trimmed at the 5` by 30 nucleotides (nt) with no truncation at the 3` ends, maximum expected error set to three, and all other options at default. After chimera detection, a total of 19 amplicon sequence variants (ASVs) were identified from 208,584 reads. ASV representative sequences were classified taxonomy using the UNITE 8.0 [24] reference database general FASTA in DADA2. Taxonomic bar plot was generated using PhyloSeq [25].

3. Results and Discussion

Overall, under non-sterile and heterotrophic conditions, microalgae cultures achieved significantly lower levels of nutrient removal than the microalgae/yeast or yeast cultures (Figures 1, 2 and 4). The COD in wastewater alone (Table 1) produced very low levels of biomass for all cultivations, however, the addition of up to 10 g/L of glucose maximised both biomass yields and nutrient removal. Although glucose was employed to increase the COD loads, a real application of this technology would envisage the use of organic food waste or wastewater with higher COD loads. Simple sugars such as glucose and fructose needed for heterotrophic growth, could be derived from pre-treating organics via saccharification. Mexico City alone generates 12,500 tonnes of municipal solid wastes a day, 50% of which is organic [26]. In addition to municipal waste, a vast amount of industrial organic waste is produced in the coun-

try. Agave is grown extensively in Mexico and is used in tequila production, the residue produced from the process is agave bagasse, 352,200 tonnes of which is generated by the industry per year [27]. The refining of sugarcane, another major crop grown throughout Mexico, also generates 15 million tonnes of organic residue annually [27]. Despite the requirement of an additional carbon source, all heterotrophic systems in this study provided substantial improvements in wastewater treatment performance and a fourfold reduction in cultivation time compared to autotrophic microalgae treatment of wastewater of the same source [28]. Heterotrophic microalgae biomass yields, when using 10 g/L of glucose, were lower than yeast biomass yields, under non-sterile conditions (Figures 1, 2 and 4).

3.1. Microalgae cultivation performance in wastewater

Cultures supplemented with 10g/L of glucose attained the highest mean biomass concentration of 0.98 ± 0.10 g/L as shown in Figure 1B. Resulting p-values from comparing TSS produced in 1, 5 and 10 g/L of glucose to 0 g/L of glucose were 0.46, 0.11 and 0.04, respectively. It can therefore be inferred that only 10 g/L of glucose was sufficient to produce a significant increase in TSS concentration compared to wastewater alone. Wastewater treatment performance was also greatest in cultures supplemented with 10g/L of glucose achieving mean nitrate, TAN and orthophosphate removals of 60, 53 and 46% respectively as shown in Figure 1A. However, this is considerably lower than the minimum reductions in total nitrogen and phosphate concentrations of 70 and 80% required to meet the standards outlined in the European Urban Wastewater Treatment Directive [29]. The maximum biomass concentration of 0.98 ± 0.1 g/L was significantly lower than the 3.46 g/L reported in literature for *Scenedesmus* sp. supplemented with 10 g/L of glucose [4]. This is likely due to the use of non-sterile wastewater in this work, which could have resulted in excess bacterial growth. As bacterial cells are typically smaller than

microalgae, several could pass through with the bulk fluid rather than being retained by the 1.6μm filters used during the TSS analysis.

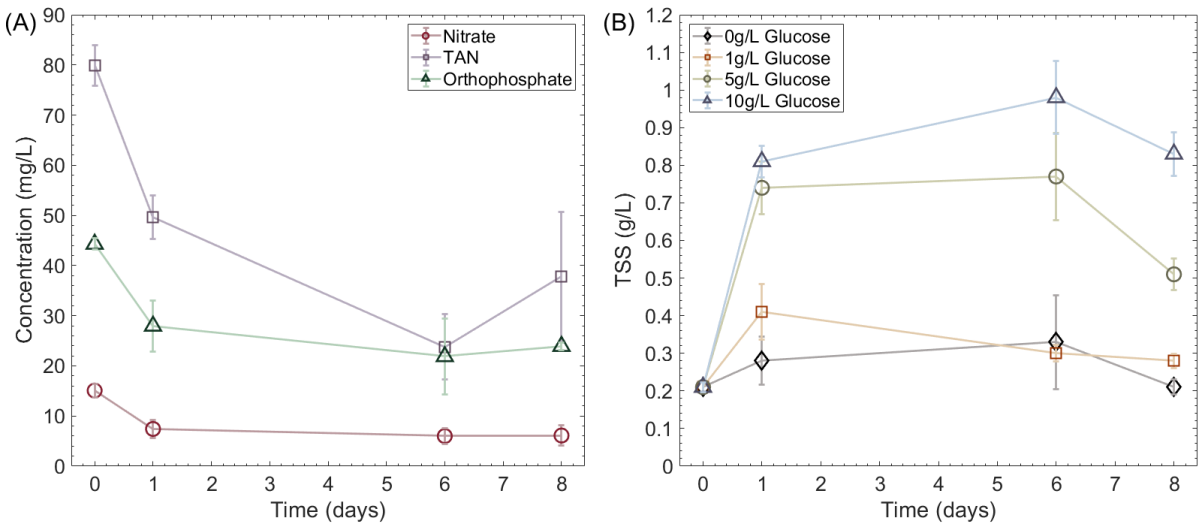

Figure 1: ***Microalgae cultivation in wastewater****. (A) Plot of mean experimental nutrient (nitrate, TAN and ortho-phosphate) concentration ± standard deviation for microalgae cultures supplemented with 10 g/L of glucose. (B) Plot of mean experimental TSS concentration ± standard deviation for microalgae cultures supplemented with 0, 1, 5 or 10 g/L of glucose. Measurement at day six for 1 g/L glucose does not follow the general trend and may have been affected by sampling. Control reactors contained inoculum and wastewater only (0 g/L glucose). TSS concentrations reported, correspond to the microalgae dry weight. Biological experiments were run in triplicate.*

3.2. Microalgae and yeast co-cultivation performance in wastewater

The microalgae and yeast co-cultures supplemented with 10 or 20 g/L of glucose showed a high level of nutrient removal achieving 91 and 94% orthophosphate, 93 and 97% nitrate and 93 and 95% TAN removal in three days, respectively. After a further four days of culture, TAN was undetectable in all cultures indicating 100% removal. Nitrate concentration decreased slightly, however, orthophosphate concentration increased. No significant differences between the two batches were found in the mean TAN ($p=0.855$, two-tailed t-test), nitrate ($p=0.783$) and orthophosphate ($p=0.974$) concentrations. It can therefore be concluded that doubling the initial glucose concentration resulted in no significant improvement in nutrient removal. The total biomass concentration at the end of the cultivations were 1.85±0.26 to 2.74±0.43g/L for the 10 and 20 g/L cultures respectively. However, microscopic analysis of the resulting cultures

revealed that the higher glucose concentration favoured yeast and bacterial growth and the mean number of microalgae cells present was 18% lower in the 20 g/L cultures. As a result increasing the glucose concentration is unlikely to improve microalgae yields. Hence, a glucose concentration of 10 g/L was selected for subsequent growth experiments. Results for this 10g/L batch, summarised in Figure 2, show that between the first and second days, there was an increase in nitrate concentration from 48.5 ± 5.0 to 70.4 ± 9.8 mg/L. This is likely the result of nitrification by the nitrifying bacteria present in the WWTP effluent. Nitrifying bacteria are chemolithoautotrophic organisms meaning they gain their energy through the oxidation of inorganic nitrogen sources using CO_2 as a carbon source. There are two main types of nitrifying bacteria used in wastewater treatment. Ammonia oxidising bacteria oxidise ammonia to nitrite whilst nitrite oxidising bacteria are responsible for the oxidation of nitrite to nitrate. The significant increase in nitrate observed suggests that both types of bacteria were likely present in the reactors. Following the increase in nitrate concentration, a 93% decrease was observed in the subsequent 24 hours. The nitrogen concentration at 4.8 ± 1.2 mg/L was lower than the 6.1 ± 2.0 mg/L achieved by the microalgae cultures (Figure 1A) in eight days. It is therefore suspected that the significantly higher nitrogen consumption rate observed in the co-cultures could be associated with yeast growth. This theory is supported by the clear dominance of the yeast after four days of culture (Figure 3).

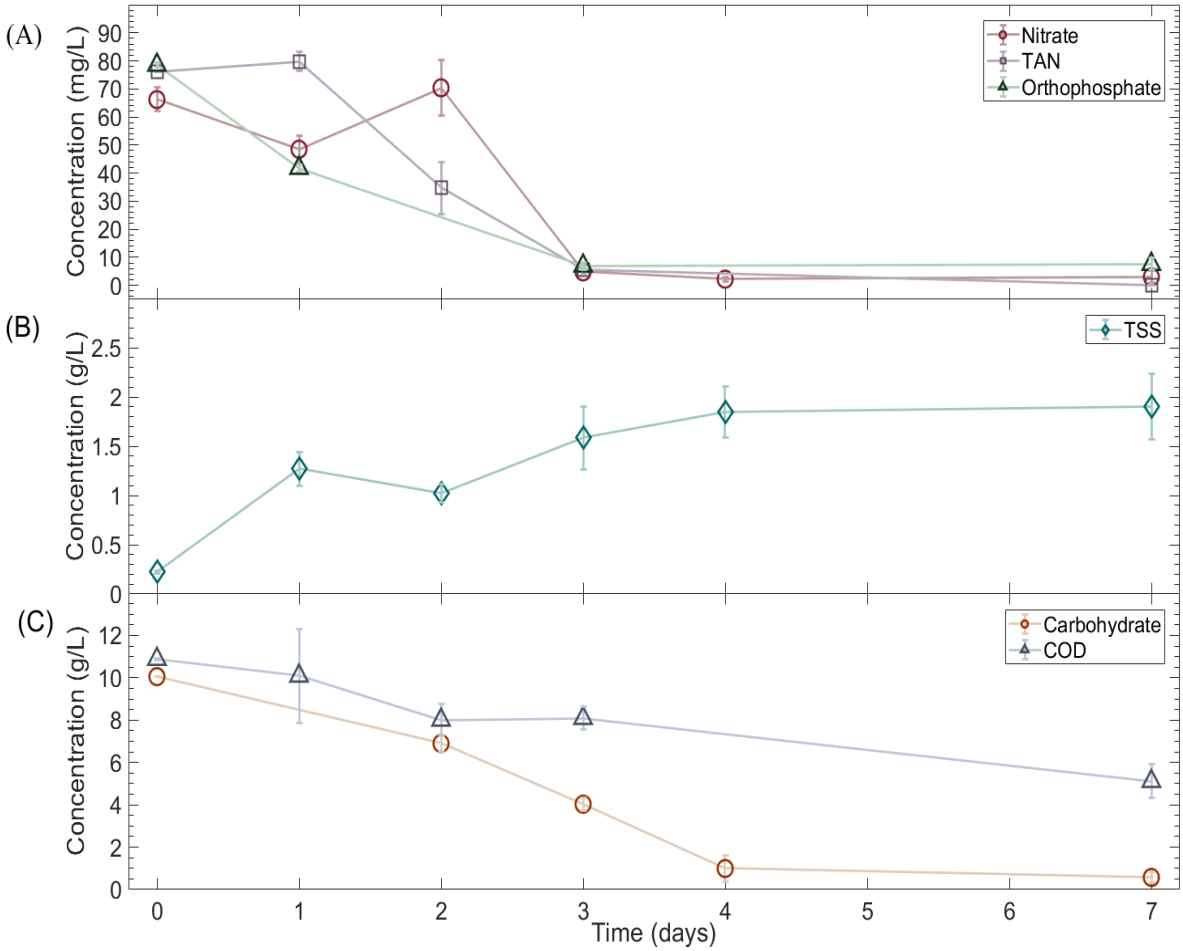

Figure 2: ***Microalgae/yeast co-cultivation in wastewater.*** *Plots of experimental nutrient (nitrate, TAN and orthophosphate) (A), biomass (B) and carbohydrate/COD (C) concentration ± standard deviation for microalgae and yeast culture supplemented with 10 g/L of glucose and air at a rate of 0.5 VVM. Biological experiments were run in triplicate*

The total yeast and microalgae biomass concentration decreased from 1.3 ± 0.2 to 1.0 ± 0.1 g/L between days one and two. The poor growth observed may be due to the sub optimal pH conditions. Under sterile conditions *Scenedesmus obliquus* have been found to grow well at pH 5-9, however, optimal performance is observed between pH 7 and 8 [30]. Yeast are able to tolerate a wide range of pH, however, acidic pH is favourable with growth being slightly reduced at pH 7 and completely arrested at pH 9 [31]. The pH was measured daily and 6N NaOH was added to raise pH to neutrality. Between measurements and daily pH adjustments, there were considerable drops to around pH 5 in all cultures. The large differences in optimal pH of the two organisms meant that optimal growth of yeast and microalgae in a single reactor may

not be feasible if a strict control mechanisms in not in place. Figure 3A shows the concentrated predominantly microalgae inoculum used to prepare the co-cultures. After just four days of culture the yeast growth became dominant as shown in Figure 3B. The poor microalgae predominance observed may have been the result of lowered pH as a result of excess yeast growth. The pH dips during growth, are characteristic of fermenting yeasts, such yeasts typically acidify their growth medium through a combination of proton pumping during nutrient uptake, direct organic acid secretion and CO_2 evolution and dissolution [32]. Interestingly, the opposite effect was observed when the non-fermenting yeast *Lipomyces starkeyi* [11] was cultivated alongside microalgae in municipal wastewater. In their case, acidification did not occur and the CO_2 product of yeast respiration was utilised in photosynthesis. Although a pH spike promoted autotropic microalgae growth and complete nutrient removal was achieved in 14 days, biomass and hence lipid accumulation were low at 300 and 45 mg/L respectively [11]. The heterotrophic co-cultures in this study achieved comparable wastewater treatment performance and greater biomass accumulation (1.9 ± 0.3 mg/L) in just seven days. TSS concentrations were also significantly higher (p value=0.004, two-tail t-test) than that of the wastewater controls (0.08 ± 0.02 mg/L).

A) B)

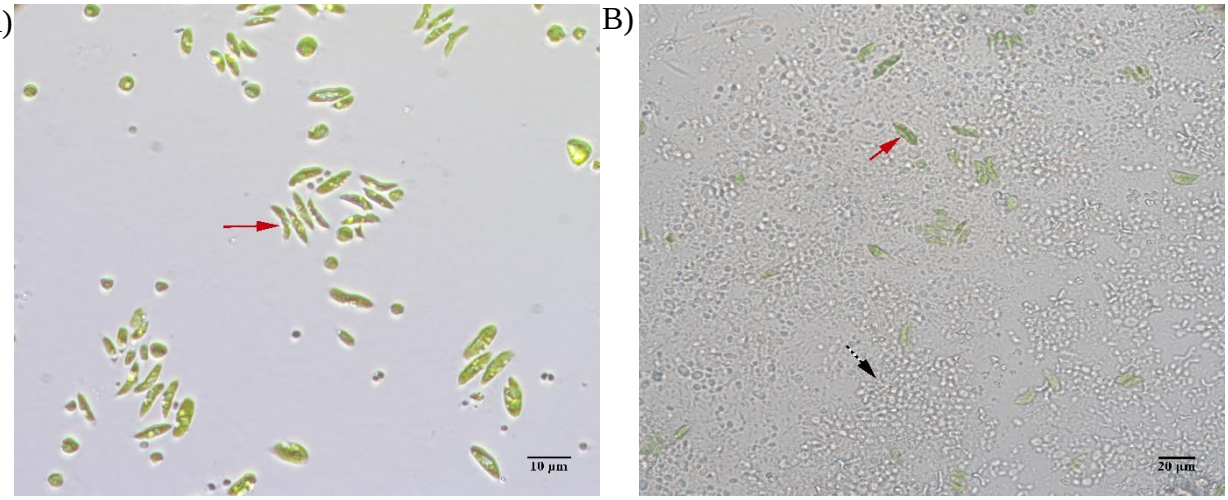

Figure 3: ***Microscope image of microorganisms in wastewater****. Microalgae inoculum (A) and microalgae and yeast co-culture supplemented with 10g/L of glucose on the fourth day of culture (B) Objective values of 100 and 63X, equivalent to total zoom values of 50 and 31.5X were used in A and B respectively. Red plain arrows indicate*

Scenedesmus sp. whilst the black dotted arrow indicates yeast. Cultures were mixed thoroughly prior to taking samples for microscopic analysis to ensure heterogeneity.

3.3. Yeast cultivation performance in wastewater

Yeast were found to thrive in the municipal wastewater successfully outcompeting bacteria under non-sterile heterotrophic conditions and produce consistently high nutrient removal in line with European standards. Results of the first two batches of triplicate yeast cultures are summarised in Figure 4. During the first three days of batch 1, 94% TAN, 90% orthophosphate, 40% nitrate, 95% carbohydrate and 50% COD removal was achieved. In the subsequent five days of culture, the nitrate and TAN concentrations decreased at a slower rate until undetectable suggesting 100% removal (Figure 4). Nitrate assimilation, a relatively uncommon characteristic in yeast, was observed in all yeast cultures. The inability of industrial yeast strains to assimilate nitrate could be the reason municipal wastewater treatment using yeast alone is yet to have been explored in detail. A slight increase in orthophosphate concentration was seen before a decrease resulting in a final removal of 93%. A final removal of 97% carbohydrates and COD was also observed following eight days of culture.

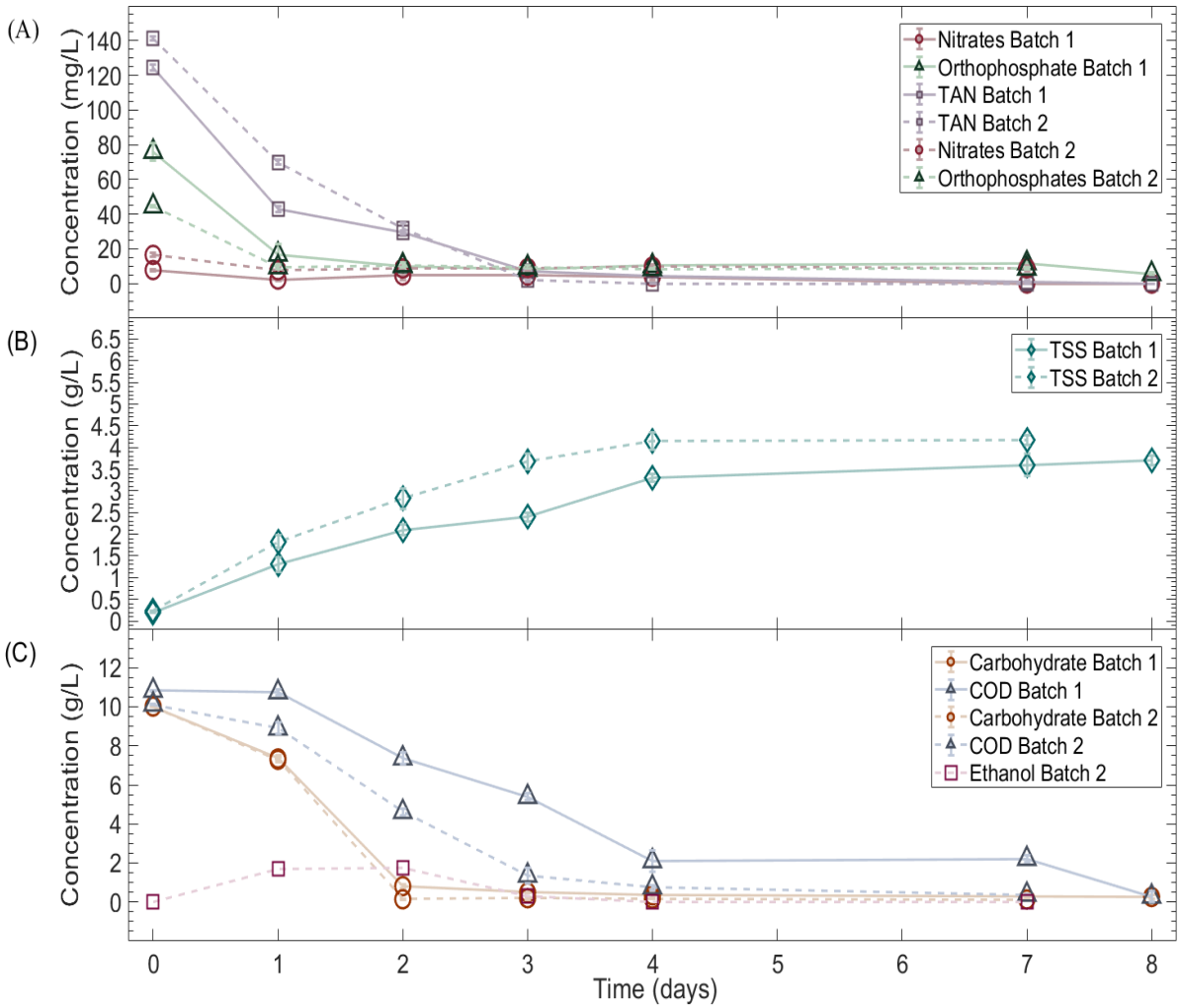

Figure 4: ***Yeast cultivation in wastewater****. Plots of experimental nutrient (nitrate, TAN and orthophosphate) (A), biomass (B) and carbohydrate/COD (C) concentration ± standard deviation for yeast batches 1 and 2 cultures supplemented with 10 g/L of glucose and air at a rate of 0.5 VVM. During batch 2 the ethanol concentration was also monitored as shown in (C)*

The metabolism of yeast is highly dependent on aeration, the nature and concentration of substrate and on the type of yeast present. Many yeast strains such as *Kluyveromyces marxianus* are Crabtree negative meaning that under aerobic conditions [33], glucose metabolism occurs via aerobic respiration only and fermentation does not occur. Some yeast strains such as *Saccharomyces cerevisiae*, however, are Crabtree positive [34]. This type of yeast typically undergoes two sequential exponential growth phases in glucose-limited cultures [34]. Crabtree positive growth was observed in this study as shown in Figure 4. An exponential growth phase was observed during the first two days of culture where biomass concentration increased from

0.2 to 2.1 ± 0.1 g/L as shown in Figure 4B. This was accompanied by a rapid depletion in nutrients (Figure 4A). During this period glucose is in abundance and the yeast cells derive their energy from fermentation through the glycolytic pathway producing ethanol and CO_2. The COD is significantly higher than the carbohydrate concentration on day two at 7363 ± 279 compared to 812 ± 103 as shown in Figure 4C. This indicates the presence of another organic carbon source (e.g. ethanol) not present at the beginning of the culture. Once the glucose supply has been exhausted, the cells enter a diauxic shift characterised by a short stationary phase during which the metabolism is switched to aerobic respiration. This can be seen for batch 1 between days two and three in Figure 4B by the slight increase in concentration to 2.4 ± 0.1 g/L. The concentration of carbohydrates decreased from 10012 ± 0.2 to 490 ± 39 mg/L in the first three days of culture as shown in Figure 4C. A second exponential growth phase occurs after this point once more than 95% of the glucose in the culture medium had been consumed. This can be seen by the rise in biomass concentration to 3.3 ± 0.1 g/L on day four in Figure 4B. Finally, a second stationary phase is reached once the COD had sufficiently depleted to 0.3 ± 0.3 g/L. In order to validate the theory that the yeast present were in fact Crabtree positive an additional batch of cultures (batch 2) was ran under the same conditions and ethanol concentration was monitored (Figure 4). Despite batch to batch variations in initial nutrient concentrations due to the nature of the culture medium, a high level of repeatability was observed as shown in Figure 4A. During batch 2, a peak ethanol concentration of 1750 mg/L occurred on the second day as shown Figure 4C, only a slight increase from the previous day's value of 1690 mg/L despite a decrease in carbohydrate concentration of 7145 mg/L. In addition, the stationary phase of growth cannot be clearly seen in Figure 4B. Carbohydrate concentration had greatly depleted by day two and exponential growth was seen between days two and four, suggesting that the diauxic shift and hence peak ethanol concentration may have occurred between the measurements taken on the first and second days.

Further growth experiments were conducted under the same conditions allowing ethanol concentration to be monitored more accurately as shown in Figure 5. There was a short lag phase within the first 2.5 hours in which ethanol concentration rose slightly from 0 to 0.3 g/L. Following this, the ethanol concentration increased exponentially to 1.6 g/L by hour six. There was then a much slower increase to the peak value of 2.5 g/L in the subsequent 42 hours.

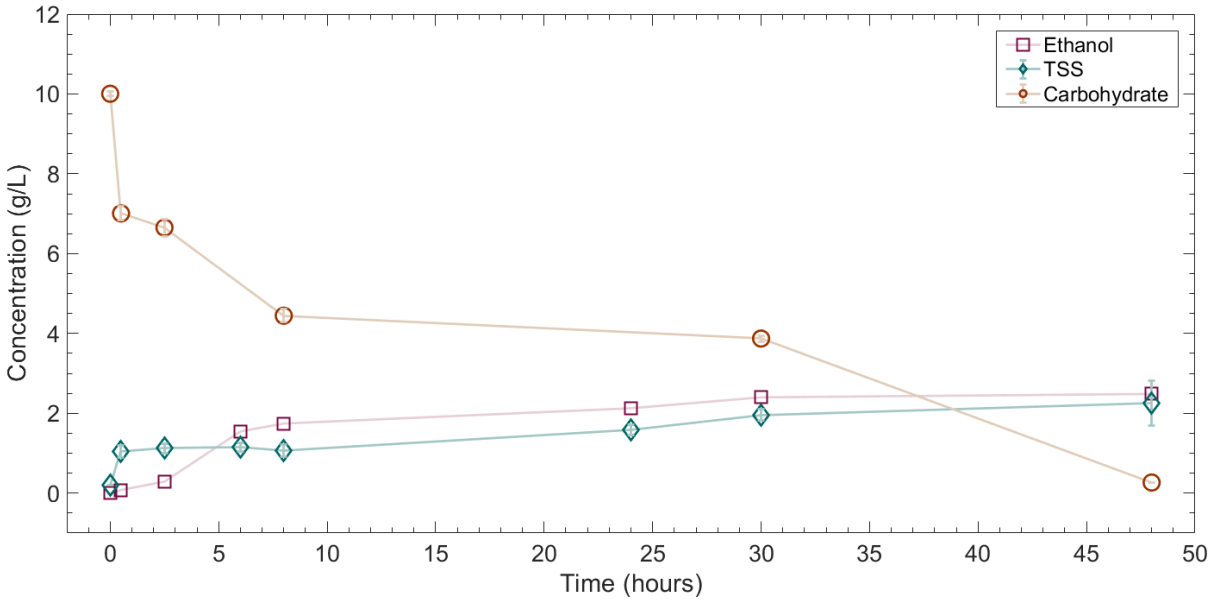

Figure 5: ***Yeast cultivation in wastewater to monitor bioethanol production.*** *Plot of experimental ethanol, biomass and carbohydrate concentration for yeast batch 3 cultures supplemented with 10 g/L of glucose.*

At the end of the 48 hours the carbohydrate concentration had depleted from 10006 ± 48 to just 266 ± 74 mg/L. In addition, between 30 and 48 hours the biomass concentration increased only slightly from 2.0 ± 0.1 to 2.3 ± 0.6 g/L suggesting that the cells were in the diauxic phase of growth as expected. The peak ethanol concentration was 2490 mg/L, corresponding to an ethanol yield of 25% by weight. This is lower than the literature yield of 32% for yeast grown aerobically in a sterile medium [34]. One possible cause of the lower than expected yield is the lack of pH control. Given the initial pH of the wastewater was around neutral which is optimal for bacteria whereas acidic pH favours yeast growth. By controlling pH in the early stages of microbial growth, bacterial growth could have been supressed and the availability of glucose

for ethanol production increased. In addition, increasing the dissolved oxygen concentration beyond 13% in *Saccharomyces cerevisiae* cultures has been found to reduce ethanol yields [35]. Excess aeration of the cultures may have also hindered ethanol production.

Phosphorous in the form of inorganic phosphate is one of the most important metabolites for all organisms [36]; it is involved in the biosynthesis of many cellular components such as ATP, nucleic acids, phospholipids and proteins. In the first two yeast batches the mean orthophosphate concentration depleted rapidly to below 10 mg/L, following this there was an increase to 11.8 ± 1.1 and 10.6 ± 1.4 mg/L in the first and second batches, respectively. The orthophosphate concentration then decreased to final values of 5.6 ± 0.9 and 8.7 ± 0.7 mg/L at the end of the eight and seven day batches, respectively. Orthophosphate concentration corresponds to the concentration of free phosphate (PO_4^{3-}) ions in the culture medium rather than the total phosphate content. Studies on yeast cells have shown that regulation of the expression of genes involved in phosphate metabolism is dependent on the phosphate concentration of the external medium [36]. The depletion of phosphate from the culture medium can result in up to a 130-fold increase in ectophosphatase activity [36]. Ectophospatases hydrolyse organic phosphates releasing additional phosphate molecules. This could have caused the slight increases in orthophosphate concentration observed in all yeast experiments.

TAN removal by the yeast was very efficient with over 90% removal in the first three days and 100% removal by the end of the two batches. Nitrification was much less prominent in the yeast cultures than the equivalent co-cultures. This may have been due to a low pH. The two commonly used nitrifying bacteria, *Nitrosomonas* and *Nitrobacter* perform nitrification over a pH range of 6.5-10 [37]. The pH of the yeast cultures dropped to around 4.5, significantly lower than the minimum tolerable pH of 6.5 which is likely to have inhibited nitrification. Also, as most bacteria thrive at neutral pH, the low pH as a result of acidification by the yeast cells is

likely to have supressed bacterial growth in the yeast cultures. This is desirable as excess bacterial growth may hinder nutrient removal and recovery of the yeast cells following treatment. The yeast presented good flocculation properties following treatment of the wastewater. Following cultivation contents of the bioreactors were stored at 4^{o}C for three days prior to yeast extraction. Within 24 hrs of storage time, bulk sedimentation occurred facilitating 74% separation of the yeast cells without centrifugation.

As yeast was found to be present in the municipal wastewater used, reactors containing just wastewater supplemented with 10 g/L of glucose were monitored to determine the effect of initial inoculation. The control culture achieved nitrate, TAN and orthophosphate removals of 73, 78 and 86% respectively in seven days. However, in this case the yeast struggled to compete with bacteria. A two-tailed two-sample t-test detected a significant difference between the means of the biomass concentrations observed for the inoculated and control cultures ($p=0.05$). However, there were no significant differences observed in the TAN ($p=0.572$), nitrate ($p=0.374$) and orthophosphate ($p=0.423$) removals. Whilst inoculating the cultures did not result in a significant increase in nutrient removal, it did prevent excessive bacterial growth. The large amount of bacteria present in the control cultures was undesirable as it resulted in a lower yield of yeast and may hinder cell recovery. As the yeast cells could generate a higher revenue than low-value bacterial biomass this would also likely reduce the profitability of the process. The addition of a small amount of yeast to the culture medium (0.2 g/L) resulted in a 175% increase in final yeast biomass concentration.

After 48 hours of growth, the mean biomass concentrations of each of the three yeast batches were 2.1 ± 0.1, 2.8 ± 0.2 and 2.3 ± 0.6 g/L, respectively (Figures 4-5). A test for equal variances

on the final TSS concentrations of the three batches showed that there was no significant difference ($p = 0.881$), meaning high reproducibility of yeast yields obtained when using different batches of non-sterilised wastewater.

3.4. Yeast identification and use for wastewater treatment

The most abundant fungal taxonomic group identified in ITS sequencing was the *Saccharomycetes* species *Barnettomyza californica*, which accounted for 45.4 % of the total abundance of the sample (Figure 6). The *Saccharomycetes* species *Cyberlindnera subsufficiens* accounted for 29.3%, whilst a *Candida* sp. accounted for 23.5%. Since the Candida sequence did not classify to species level, a BLAST search of the representative sequence was performed, which resulted in significant alignments with *Scheffersomyces spartinae, Candida thasaenensis, Candida gosingica*, all with a score of 388 at 80% query coverage and 98.6% identity. No other representative sequence accounted for more than 0.1% of abundance. *Barnettomyza californica* and *Cyberlindnera subsufficiens*, the two species which accounted for around 75% of abundance are both known to be capable of both glucose fermentation and nitrate assimilation [38].

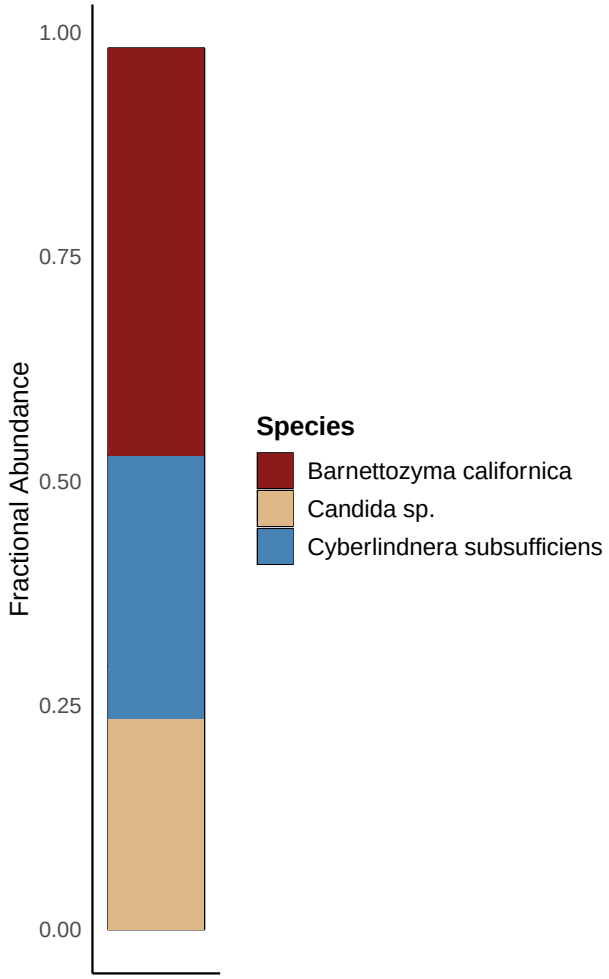

Figure 6: ***Yeast Identification.*** *Stacked bar chart of fractional abundance of the three representative sequences that accounted for 98.2% of the total sequenced community, as derived from DADA2. The size of each section of the bar reflects the relative fractional abundance of a given representative sequence as a proportion of the total read count for the three sequences. Red denotes Barnettozyma californica (45.4%); blue denotes Cyberlindnera subsufficiens (29.3%); beige denotes Candida sp. (23.5%). No other representative sequences accounted for > 0.1% abundance and so were excluded from this figure.*

Wild wastewater yeast cultivations present a relatively low cost means of treating municipal wastewater. With careful selection of the microorganisms present there is potential to greatly improve yields of the desired biofuel. The use of a non-fermenting nitrate assimilating yeast such as *Lipomyces starkeyi* could improve nutrient removal without the acidification effect associated with fermenting yeasts. This would allow alkaline pH conditions to be maintained promoting microalgal growth and lipid accumulation. On the other hand, increasing the proportion of brewing yeasts in the fermentation medium would maximise bioethanol yields.

4. Conclusions

An investigation into the performance of novel non-sterile heterotrophic systems to remove nutrients and organic matter from municipal wastewater has been conducted. Glucose supplementation was necessary in this study to deplete nutrients and increase biomass yields, hence the system proposed could be of interest to the industrial sector providing a sustainable means of treating wastewaters with high organic loads or pre-treated organic waste. Microalgae were unable to compete with proliferating microorganisms, achieving relatively low biomass concentrations along with poor levels of nutrient removal. Co-culturing microalgae with yeast led to poor yields of both microalgae and yeast mainly due to pH fluctuations, however, excellent levels of nutrient removal were achieved. The wild yeast consortium found in the municipal wastewater produced the most promising results, achieving consistently high biomass concentrations and nutrient removal rates. Here, nitrate assimilation, a relatively uncommon characteristic in yeast, maximised total nitrogen removal in the yeast cultures; and the Crabtree positive metabolism of the wild yeast consortium highlighted the possibility for simultaneous wastewater treatment and bioethanol production. With this, our study showed the potential for generating useful yeast biomass and bioethanol whilst achieving wastewater biological treatment targets. As the application of the yeast consortium for municipal wastewater treatment is a novel concept, this study focussed on characterisation and species identification to provide a foundation for future in-depth optimisation studies.

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574 **Declarations of interest**

575 None.

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